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Fruit ripening mutants yield insights into ripening control

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Fruit ripening is a developmental process that is exclusive to plants whereby mature seed-bearing organs undergo physiological and metabolic changes that promote seed dispersal. Molecular investigations into ripening control mechanisms have been aided by the recent cloning of tomato ripening genes that were previously known only through mutation. Advances in the genomics of tomato have provided genetic and molecular tools that have facilitated the positional and candidate-gene-based cloning of several key ripening genes. These discoveries have created new inroads into understanding of the primary ripening control mechanisms, including transcription factors such as those encoded by the *RIPENING-INHIBITOR* (*RIN*) MADS-box and *COLOURLESS NON-RIPENING* (*CNR*) SPB-box genes, which are necessary for the progression of virtually all ripening processes. They have also facilitated the elucidation of downstream signal transduction components that impact the hormonal and environmental stimuli that coordinate and modulate ripening phenotypes.

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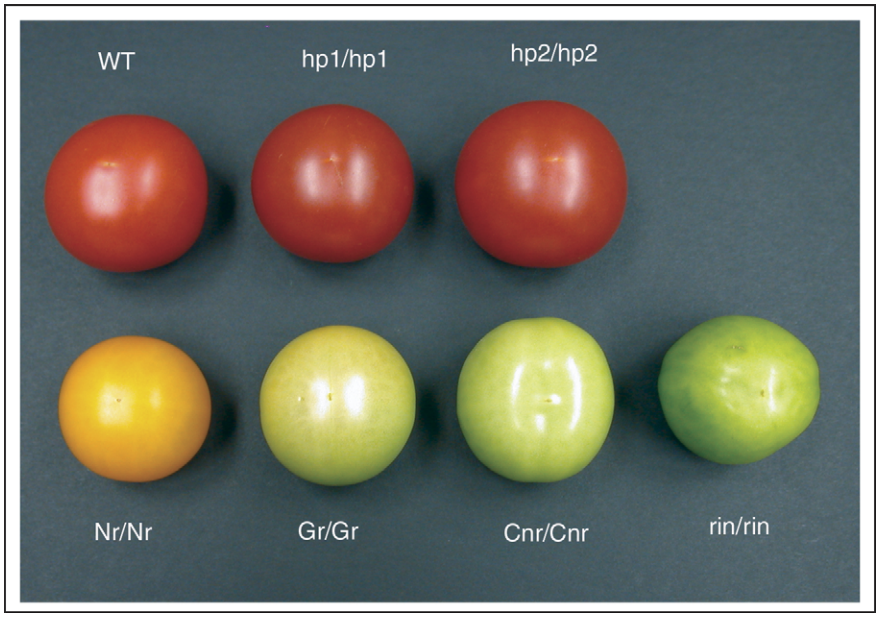
Introduction

Fruit constitute important components of human and animal diets, and are organs and developmental systems that are unique to plants. As a result, considerable scientific study has focused on questions of fruit organogenesis, differentiation, development and maturation, in addition to the genetic basis of organoleptic and nutritional qualities (reviewed in [1–3,4•]). Pioneering work on the genetic basis of fruit formation and development has emphasized the model system *Arabidopsis* [5], whereas investigations of organ expansion, maturity, ripening, shelf-life and nutritional quality have centered on the crop model tomato (*Solanum lycopersicum*) [2]. Tomato, the centerpiece of the Solanceae family, has emerged as a model of fleshy fruit development, primarily because this is the species for which the genetic and molecular toolkits are most advanced. Extensive germplasm collections, well-characterized mutant stocks, high-density genetic maps, immortalized mapping populations, efficient transient and stable transformation, deep expressed sequence tag (EST) resources, microarrays and an ongoing genome sequencing effort all contribute to the utility of this experimental system (see www.sgn.cornell.edu and www.tigr.org for links to these resources). Well-characterized ripening mutations, short generation time, a long history of physiological, biochemical and molecular investigations related to fruit development and maturation, and interest in the species as an important commodity crop, have fueled considerable effort on understanding ripening in tomato. Early molecular analyses of fruit ripening focused on the roles of cell-wall-metabolizing and structural proteins [6], and on the genetic basis of ethylene synthesis [7]. In recent years, the molecular biology of ripening has turned to genomics approaches to reveal insights into primary ripening control upstream of ethylene, ripening-related signal transduction systems and downstream metabolic networks. These advances have been facilitated by increasingly efficient positional cloning in tomato, by the development of a model for ethylene signal transduction from *Arabidopsis* (which could be tested in tomato) and by improved metabolic profiling technologies, respectively. The result has been the opening of a new frontier in ripening molecular biology that is focused on upstream transcriptional control and on the characterization of hormonal and environmental signaling mechanisms.

Revealing the secrets of ripening mutants

Deep screens for ripening mutants have been limited by the number of mature tomato plants that can be reasonably managed in field trials. For example, the screening of 10 000 tomato M_2 families consisting of twenty individuals per family would require approximately 20 hectares (50 acres) of field space. A mutant screen on this scale has recently been performed, rendering numerous mutant lines available for genetic purification and analysis [8]. Previously characterized ripening mutants have otherwise reflected mostly spontaneous mutations or wild allele variants that have been recovered from production fields or in breeding programs. These include pleiotropic ripening mutations, such as *Colorless non-ripening* (*Cnr*), *ripening-inhibitor* (*rin*), *Never-ripe* (*Nr*), *Green-ripe* (*Gr*) and *high-pigment* (*hp-1* and *hp-2*) (Figure 1), all of which have now been isolated either by positional cloning or by genetic mapping of mutant loci and candidate genes.

Figure 1

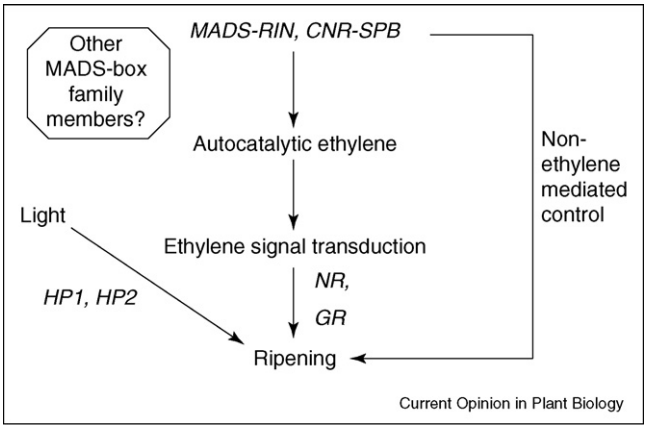


Normal and mutant tomato fruit. Normal tomato cultivar Ailsa Craig ripe fruit ten days post breaker and equivalent age fruit homozygous for the *high-pigment 1* (*hp1/hp1*), *high-pigment 2* (*hp2/hp2*), *Never-ripe* (*Nr/Nr*), *Green-ripe* (*Gr/Gr*), *Colorless non-ripening* (*Cnr/Cnr*) and *ripening-inhibitor* (*rin/rin*) mutations.

Insights into the transcriptional control of fruit ripening

The *rin* and *Cnr* mutations are recessive and dominant mutations, respectively, that effectively block the ripening process and result in mutant fruit that fail either to produce elevated ethylene or to respond to exogenous ethylene by ripening [9,10••]. The *rin* mutation is additionally noteworthy because hybrids (*rin/Rin*) form the basis for most present-day production of slow-ripening, long shelf-life, fresh-market tomatoes. Both the *rin* [9] and *Cnr* [10••] loci encode putative transcription factors, providing the first insights into dedicated fruit-specific transcriptional control of ripening. *rin* encodes a partially deleted MADS-box protein of the SEPELATTA clade [11], whereas *Cnr* is an epigenetic change that alters the promoter methylation of a SQAMOSA promoter binding (SPB) protein. The relative placement of these proteins in the fruit maturation regulatory network remains unclear (i.e. whether they operate independently or in the same pathway). Both are necessary, however, to induce ripening-associated increases in respiration and ethylene concentration, characteristics typical of tomato and other so-called climacteric fruits. Furthermore, the fruit of both mutants are responsive to ethylene, as shown by monitoring ethylene-inducible genes, but neither ripens in response to this hormone, suggesting that both genes lie upstream of crucial ripening activities (including ethylene production) and have some functions that are ethylene

Figure 2



Summary of ripening control. The ripening-specific transcription factors MADS-RIN and CNR-SPB are necessary for the induction of ethylene- and non-ethylene-mediated ripening control, as defined by the *rin* and *Cnr* mutations, respectively. The ethylene signal is transduced although the ethylene receptor (*NR*) and the GR protein, which might participate in maintaining receptor-copper homeostasis. These genes were defined by the cloning of the alleles that are responsible for the *Nr* and *Gr* mutations, respectively. *HP1* and *HP2* were isolated by cloning of the *hp1* and *hp2* mutant alleles, respectively, and encode tomato homologs of the *Arabidopsis DDBP1* and *DET1* light signal transduction genes, respectively. Additional MADS-box genes that are expressed in ripening fruit (Table 1) represent additional candidates for the control of ripening-related transcription.

independent (Figure 2). This non-ethylene-mediated aspect of ripening physiology is intriguing because it suggests that both the Rin MADS and Cnr SPB proteins could be candidates for ripening regulators that are conserved among fruit that demonstrate either climacteric or non-climacteric ripening physiologies. Indeed, the most widely studied non-climacteric ripening system is strawberry, which increases neither respiration nor ethylene production during ripening, and is largely unresponsive to inhibitors of ethylene action such as 1-methylcyclopropene (1-MCP) [12]. Consistent with a theory in which such regulators might be widely conserved, a ripening-related strawberry homolog of the tomato *RIN* gene has been isolated [9].

Characterization of MADS-box genes in *Arabidopsis* and additional species shows that MADS-box proteins often function as heterodimers or multimers [13]. Consequently, additional MADS-box proteins might be expected to play roles in fruit ripening. Examination of the tomato EST collection indicates that at least 13 additional MADS-box genes are expressed in ripening fruit, and eight show increased or moderate/high expression in this tissue (Table 1). Microarray and EST abundance analysis indicates 15 or more putative transcription factor genes whose expression parallels ripening, including a number whose expression is not influenced by ethylene [14•,15]. Comparative *in silico* expression profiling using public grape EST abundance data reveals only three putative transcription factors that are conserved between tomato and grape [15]. This is perhaps not unexpected as tomato and grape exhibit vastly different ripening programs and physiologies. Grape is a non-climacteric fruit that undergoes ripening (termed veraison) in parallel to fruit expansion and seed maturation. By contrast, climacteric tomato fruit ripen only after expansion is complete and seed are mature. The fact that tomato and grape appear to share a subset of common ripening regulators might suggest that these genes represent primary functions that have been conserved through evolution for ripening control. As such, these genes represent priority candidates for further functional analysis.

The molecular basis of the ethylene response in fruit

Recent efforts related to the molecular biology of the ethylene response of ripening fruit have centered on the characterization of tomato homologs of *Arabidopsis* ethylene signal transduction genes. All of the analyzed components of the *Arabidopsis* ethylene signaling machinery are clearly conserved in tomato [16–20], but family sizes and expression profiles are differ between some *Arabidopsis* and tomato ethylene signaling genes. Tomato harbors six ethylene receptors compared to the five in *Arabidopsis* [21], but ethylene-binding studies indicate similar ethylene affinities for all tomato and *Arabidopsis* receptor proteins [22•]. Nevertheless, gene expression studies reveal distinct expression and regulatory profiles for tomato and *Arabidopsis* ethylene receptor (*ETR*) genes, including the transcriptional ability of tomato *LeETR4* to compensate for the transgenic repression of *NR* mRNA levels [23]. The *CONSTITUTIVE TRIPLE RESPONSE1* (*CTR1*) MAP kinase kinase kinase is represented by a single protein in *Arabidopsis*, through which all ethylene signaling is transduced. By contrast, at least three *CTR1* genes that have distinct expression patterns are found in tomato, all of which demonstrate the ability to complement *Arabidopsis ctr1* loss-of-function mutations [19].

Most of the ethylene signaling activities defined to date were originally identified through elegant seedling screens performed in *Arabidopsis*, but a novel ethylene signaling function was recently revealed by the simultaneous cloning of the tomato *GR* and *Arabidopsis REVERSION TO ETHYLENE SENSITIVITY1* (*RTE1*) loci. *GR* was cloned by positional cloning of the gene underlying a dominant ripening mutation [24••], whereas *RTE1* was cloned from a second-site suppressor screen of an *Arabidopsis* ETR1 receptor mutant [25•]. The biochemical nature of the GR and RTE1 proteins remains unclear, but their amino acid sequences suggest membrane localization and possible copper-binding activities. As the ethylene receptors require Cu^{2+} for activity and have been localized to membranes of the endoplasmic reticulum [26,27], it is possible that the GR and RTE1

Table 1

MADS-box genes expressed in ripening tomato fruit.

GenBank accession	Homology	Ripe fruit expression
XP_483124	MADS *Oryza sativa* (japonica cultivar group)	Low
AAM15775	MADS-RIN (*S. lycopersicum*)	Moderate
AAP57412	MADS-box protein 1	High
AAF22139	MADS box protein (*Capsicum annuum*)	High
CAC83066	MADS-box protein	High
AAM15774	MADS-MC tomato AP1 homolog	Low
AAP57413	MADS-box protein 5	Low
AAF77579	MADS-box protein (*C. annuum*)	High

proteins are involved in receptor-copper homeostasis. Tomato harbors at least two additional GR-family members, *GR-like 1* (*GRL1*) and *GRL2*, whereas only one homolog of RTE1 (termed RTH) is found in the *Arabidopsis* genome.

Characterization of the *Gr* mutation revealed that the deletion of 334 nucleotides spanning the promoter and 5′ untranslated sequences of *GR* resulted in the ectopic expression of this gene, specifically in fruit tissues and from early development through ripening. The result of this *GR* overexpression is drastically reduced fruit ethylene sensitivity, resulting in severe ripening inhibition even though normal or elevated levels of ethylene are produced by *Gr* fruit [28•]. Transgenic overexpression of *GR* using the CaMV35s promoter resulted in similar ripening inhibition but, surprisingly, in minimal alteration of ethylene sensitivity in non-fruit tissues. No measurable effects on the elongation of seedling hypocotyls, on leaf epinasty or on leaf senescence were observed, and only mild effects on pedicel abscission, petal senescence and root elongation could be detected. The predominant fruit phenotypes of transgenic *35s*::*GR* tomato lines suggest that *GR* might interact specifically with components of the fruit-specific ethylene response, and thus these lines represent a novel tool with which to explore the currently unknown nature of fruit-specific ethylene-response mechanisms. Transgenic knockouts for definitive functional analysis of *GR*, *GRL1* and *GRL2* are currently being generated (C Barry, J Giovannoni, unpublished). This is especially important as mutant and transgenic data both represent insights gained from ectopic expression.

Genetics of fruit chemistry

Biochemical pathways that impact fruit quality and nutritional content have long been the focus of ripening biology. Indeed, many steps in carotenoid biosynthesis have been elucidated through the study of tomato mutations and genes [29–32]. The genetics of downstream cleavage activities that result in signaling molecules, growth regulators and aroma volatiles that might serve as nutritional signals for seed-dispersing organisms have been reviewed recently [4•,33]. Emerging metabolic profiling technologies, combined with the use of structured and immortalized mapping populations, have permitted the mapping of large numbers of fruit metabolite quantitative trait loci (QTLs). For example, QTLs that correspond to candidate carotenoid synthesis genes have been distinguished from those that might represent novel regulatory loci [34••]. Although genes encoding the majority of steps in carotenoid synthesis have been isolated, little is known of how this pathway is regulated and hence such regulatory loci are especially interesting.

Recent insights into the regulation of carotenoid accumulation in fruit have resulted from cloning of the phenotypically identical *hp1* and *hp2* loci. In addition to elevated flavonoid and carotenoid profiles in mature ripe fruit, both mutations result in short, anthocyanin-accumulating hypocotyls and high chlorophyll content in leaves (and in unripe fruit). Both genes encode tomato homologs of previously described nuclear-localized *Arabidopsis* light signal transduction proteins: HP2 is homologous to DETIOLATED1 (DET1) [35] and HP1 to DAMAGED DNA BINDING PROTEIN 1 (DDB1) [36]. In *Arabidopsis*, *DET1* and *DDB1* encode constituents of the same protein complex [37], which might play a role in chromatin remodeling in response to light [38]. This interaction probably explains the strong similarity between the phenotypes of *hp1* and *hp2* mutants, which at their core reflect heightened sensitivity to normal light regimes, presumably mediating elevated pigment levels to protect against the perceived threat of photooxidation. Transgenic tomato experiments that were designed to prove the role of light signal transduction components in regulating the accumulation of carotenoids and other antioxidants demonstrated that manipulation of both positive (tomato *CONSTITUTIVELY PHOTOMORPHOGENIC 1* [*COP1*]-*like*) and negative (tomato *ELONGATED HYPOCOTYL 5* [*HY5*]) light signaling regulators, in addition to the *CRY2* photoreceptor, had corresponding effects on carotenoid and flavonoid accumulation [36,39]. Transgenic repression of the tomato *DET1/HP2* gene, using a fruit-specific promoter, demonstrated that the desirable light-response phenotypes of elevated carotenoid and flavonoid accumulation in fruit could be separated from the deleterious results of such manipulations in non-fruit tissues, including dwarfism and reduced yield [40].

The ripening-associated accumulation of nutritional metabolites such as sugars, organic acids, carotenoids, flavonoids and ascorbate represents one of the many reasons for interest in the tomato ripening system. Interestingly, other important nutritional pathways are specifically downregulated during ripening. For example, folic acid (vitamin B9) cannot be synthesized *de novo* by humans and must be acquired in the diet. Folate is typically found in green plant tissues and is moderately abundant in green tomato fruit, although its synthesis and accumulation is substantially repressed as the fruit transitions to maturation [41]. Folate is synthesized from pteridine, p-aminobenzoate (PABA) and glutamate precursors, and the expression of genes that are involved in pteridine and PABA synthesis is known to drop dramatically in concert with induction of ripening [42,43]. PABA pools remain stable during ripening, suggesting that pteridine synthesis might be rate limiting in fruit folate accumulation. This hypothesis was verified by elevated pteridine and associated folate synthesis in tomato fruit that expressed a synthetic GTP cyclohydrolase-1 gene, which encodes the first step in pteridine production [44]. Elevated pteridine synthesis shifted the limitation for further folate synthesis to PABA, as shown by subsequent

elevation of folate levels when transgenic fruit were supplied with exogenous PABA.

Direct regulators of pathways that are responsible for the synthesis of important fruit metabolites, including carotenoids and folate, remain to be identified, but the development of tomato germplasm and genomics resources hold promise for the identification and isolation of such loci in the near future. For example, the use of introgression lines from wild tomato species has facilitated the mapping of numerous carotenoid-accumulation QTLs. The underlying regulatory genes will no doubt include at least some components of light signal transduction. Comparative profiling of the transcriptomes of *hp1*, *hp2* and corresponding normal near isoclines will probably provide candidate genes for these loci, as will the ongoing tomato genome sequencing effort (www.sgn.cornell.edu). Such approaches should lead to identification of candidate genes for large numbers of the developmental, metabolic and fruit quality loci that have been recently placed on the tomato genetic map [34••]. These approaches will also foster the localization of additional loci of interest including, for example, folate accumulation QTLs, and will most certainly help to drive future ripening research in the directions of primary ripening regulators and downstream effectors.

Conclusions

Physiologically characterized single gene tomato ripening mutants, which in some cases have been available for decades, have recently become accessible at the molecular level as the genomics infrastructure for tomato has expanded. Cloning of the *RIN* and *CNR* genes defined the first ripening-specific transcription factors and provided insight into ripening control upstream of ethylene. The *Nr* mutation revealed an ethylene receptor gene, and *Gr* has been found to encode a novel component of ethylene signaling that might impact receptor-copper homeostasis in a largely fruit-specific manner. The presence of two additional *GR*-like genes in tomato remains to be explained, although the fruit specificity of *GR* would suggest possible similar ethylene signaling functions for non-fruit tissues. Signal transduction systems that might fine tune ripening phenomena according to environmental conditions are poorly understood, although the characterization of the *HP1* (*DDB1*) and *HP2* (*DET1*) genes represent initial inroads into dissecting regulatory pathways that impact a subset of fruit characteristics, including those that contribute to metabolic content and associated fruit quality. The first deep screen of mature mutagenized tomato plants, combined with extensive fruit metabolic QTL mapping, holds the promise of numerous additional functionally defined loci that will become increasingly accessible as part of expanding genomics resources and the emerging tomato genome sequence. The initial inroads into primary ripening control and signal transduction made in recent years are certain to be expanded substantially in those that follow.

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